MURINE B CELL DIFFERENTIATION LINEAGES

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Two-color immunofluorescence studies with the fluorescence-activated cell sorter (FACS)[1] distinguish three populations of mouse B cells according to the amounts of surface IgM and IgD expressed per cell (1–3). Characteristic strain and organ distributions further define these populations. Thus, a population, termed I, that expresses high IgD and intermediate IgM levels is the most numerous in most mice but is missing in mice carrying the CBA/N X-linked immunodeficiency. A population, termed II, that expresses high IgD and high IgM levels is found in comparable frequencies in all strains; and a population, termed III, that expresses low IgD and high IgM levels is found in spleen but not in lymph nodes and occurs at higher frequencies in xid and autoimmune mice than in normal mice. These populations are diagrammed on the IgM, IgD staining plots in Fig. 3.

Populations I, II, and III differentially express a series of lymphocyte cell surface proteins including Ia, ThB and, very surprisingly, the pan-T cell antigen Ly-1 (2–4). Ly-1, formerly called Lyt-1, is present on a unique subpopulation (Ly-1 B) that comprises most of the cells in population III (except in CBA/N mice) and is greatly expanded in NZB-related autoimmune mice (3–5). Functional studies with sorted Ly-1 B cells show that these cells are solely responsible for the "spontaneous" IgM secretion peculiar to cultured NZB spleen cells (4). IgM antibodies secreted in response to antigenic stimulation, in contrast, are produced by different spleen cells that carry neither Ly-1 nor IgD.

The distinctive surface characteristics, functional properties, organ location, and development pattern demonstrated for Ly-1 B cells suggest that this small subpopulation (and consequently much of population III) represents a separate lineage of B cells (5). The lineage relationships of the remaining B cells (which constitute the vast majority of the Ig-bearing cells), however, are not well understood. There are two major theories concerning the lineage distinctions among these cells (6, 7): (*a*) the single-lineage concept in which a pre-B cell population proceeds through several stages of increasing "maturity" and termi-

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[1] *Abbreviations used in this paper:* AP, allophycocyanin; Bi, biotin; Con A, concanavalin A; D-K, dinitrophenyl-keyhole limpet hemocyanin; FACS; fluorescence-activated cell sorter; Fl, fluorescein; LPS, lipopolysaccharide; PE, R-phycoerythrin; PFC, plaque-forming cell; PI, propidium iodide; PNA; peanut agglutinin; SE, sheep erythrocyte; SMPB, succinimidyl-4-(*N*-maleimidophenyl)butyrate; SPDP, *N*-succinimidyl 3-(2-pyridyldithio)propionate; TR, Texas Red.

nates in antibody-secreting cells (or memory cells); and (*b*) the branched lineage concept in which a precursor population divides into two (or more) branches that have functionally distinct response properties.

The argument for multiple B cell lineages is based on studies showing that the B cells responding to certain types of antigens are distinguishable (8) and that, during neonatal development, responses to certain antigens are lower in comparison with adults (9). In addition, work with CBA/N mice, which fail to respond to a subset of T-independent (type 2) antigens (10–12) suggests that these mice lack a subset of B cells that express specific cell surface determinants (13–15) known as Lyb-3, Lyb-5, and Lyb-7. These mice clearly have fewer B cells and indeed are missing the majority type mature population found in normal mice defined by the correlated expression of IgM and IgD (1–3); however, the question remains as to whether this deficit represents a maturational block in a single lineage or the complete deletion of a separate lineage. More recent work (16, 17) with mice that have both xid and nude (nu/nu) defects has shown that the combined defect results in profound decreases in the serum levels of all Ig isotypes assayed and in the number of Ig-bearing cells in the lymphoid tissues (compared with either nude or xid mice). The data from these later studies were interpreted as favoring (but not proving) a branched lineage model (16, 17).

In this study, we addressed this B cell lineage problem with the methods that we had used to define and characterize the Ly-1 B population: quantitative multiparameter analyses and sorting with fluorochrome-labeled monoclonal antibody reagents. We first characterized the reactivities of two monoclonal antibodies that label subpopulations of splenic B cells and then used correlated three- and four-color immunofluorescence analyses (18, 19) with these and other monoclonal reagents to compare normal, xid, and nude mouse B cells. The results show that these monoclonal reagents recognize distinct antigens expressed on functional cell populations, consistent with the suggestion (16, 17) of two separate lineages of B cells (in addition to Ly-1 B), one of which is missing in xid mice but not in nude animals and the other which is diminished in nude animals but not in xid mice.

Materials and Methods

Mice. CBA/H-T6 and CBA/N, provided by Dr. J. J. Haaijman, Institute of Experimental Gerontology, TNO, The Netherlands, were bred at our animal facilities. BALB/c mice were also bred in our facility. CBA nu/nu mice were generously provided by Dr. H. Wortis, Tufts University, Boston, MA and Dr. I. L. Weissman, Stanford Medical School, Stanford, CA.

Monoclonal Antibodies. Rat anti-mouse IgM (clone 331-12) was produced by Dr. P. W. Kincade (20). Mouse monoclonal anti-IgD (specific for the a allotype, Igh-5a) and rat monoclonal antibodies 30-E2 and 53-10.1 were all produced and characterized in this lab (21, 22).

Fluorochrome Coupling. Biotin (Bi) and fluorescein (Fl) labeling procedures have been described elsewhere (23, 24). Reagents were conjugated at low (<3) label/protein ratios to minimize background staining. Texas Red (TR)-labeled avidin and peanut agglutinin (PNA) were prepared as described previously (4) using sulfarhodamine obtained from Molecular Probes Inc., Junction City, OR. All reagents were stored at 4°C with 0.1% sodium azide and were deaggregated at 100,000 *g* for 10 min in a Beckman Airfuge (Beckman Instruments, Inc., Fullerton, CA) immediately before use.

Phycobiliprotein Conjugates. We have previously purified to homogeneity R-phycoery-

thrin (PE) and allophycocyanin (AP) from red algae by ammonium sulfate precipitation and chromatography on hydroxylapatite, followed by chromatography on an ACA-34 (LKB, Bromma, Sweden) gel filtration column (25). The PE-avidin conjugate was prepared by cross-linking the two proteins with *N*-succinimidyl 3-(2-pyridyldithio)propionate (SPDP) (26) and was generously provided by John Kimura, Becton-Dickinson Monoclonal Center, Mountain View, CA. Phycobiliprotein antibody conjugates were prepared by labeling Ig with succinimidyl-4-(*N*-maleimidophenyl)butyrate (SMPB) (27) and then reacting this labeled protein with phycobiliprotein that had been previously thiolated with S-iminothiolane (28). SPDP, SMPB, and S-iminothiolane were all purchased from Pierce Chemical Co., Rockford, IL. Molecular weight analysis by high performance liquid chromatography (HPLC) (TSK-250 column; Bio-Rad Laboratories, Richmond, CA) showed that the conjugates consist primarily of one or two dye proteins coupled to a single IgG.

Tissue Section Staining. Brachial lymph nodes from BALB/c mice immunized with sheep erythrocytes (SE) were removed 7 d after footpad immunization with 10 million erythrocytes. Sections were prepared and stained as described previously (29), either with Bi-PNA or with Bi–anti-BLA-1; second-step horseradish peroxidase-conjugated avidin revealed the staining after visualization with diaminobenzidine. Tissue staining was carried out in the laboratory of Dr. E. Butcher.

Staining for FACS Analysis and Sorting. Single-cell suspensions (from which the erythrocytes had been lysed by 0.165 M ammonium chloride) were stained in microtiter wells as described previously (4). Green fluorescence was derived from directly labeled Fl reagents while red fluorescence was obtained by using biotinated first-step antibodies followed by TR-labeled avidin in a second incubation. Propidium iodide (PI) was added (50 μl at 1 μg/ml per well) to label dead cells (30). Larger numbers of cells (for sorting) were stained in 15-ml conical centrifuge tubes. Staining for three-color immunofluorescence was carried out similarly, except that AP-conjugated reagent was included with the fluoresceinated and biotinated reagents in the first step and PE-labeled avidin replaced TR-labeled avidin in the second incubation. Four-color staining was carried out using direct phycobiliprotein conjugates (PE and AP) together with a biotin-labeled reagent, followed by TR-avidin second-step staining. PI was omitted in three- and four-color staining.

Two-Color Immunofluorescence. All the immunofluorescence studies were carried out on an extensively modified dual-laser FACS (B-D FACS Systems, Sunnyvale, CA) equipped with logarithmic amplifiers (for the fluorescence channels) to measure light scatter (size) and the amounts of fluorochrome-labeled monoclonal reagents bound to individual cells (18). In addition, we use PI staining (measured with a third fluorescence detection system) to eliminate the few (PI-stained) dead cells that failed to be rejected by the normal scatter-gating method for excluding dead cells (30). We used a VAX computer to collect and store individual measurements on 30,000 (live) cells as list mode data for later analysis using programs developed by Mr. W. Moore of this department.

Three- and Four-Color Immunofluorescence. As has been described previously (31), it is possible to use a plant dye protein (PE) to obtain good quality two-color immunofluorescence from a single laser (at 488 nm) exciting the fluorescence of Fl (detected at 525 nm) and PE (detected at 570 nm). Electronic compensation (32) for the small overlap of Fl fluorescence on the PE detector and PE fluorescence on the Fl detector yields signals essentially identical to either reagent used alone. We have added extra beam splitters, two extra photomultiplier tube detectors, and special filters that now permit us to use the single-laser/two-color system in conjunction with a second laser (at 605 nm) that excites a second pair of immunofluorescence dyes, TR (detected at 620) and AP, a second plant-derived fluorescent protein (33) (measured at 645 nm). This system will be described in detail elsewhere.[2]

Data Analysis. Two-color staining data are presented as contour plots that can be viewed as representations of three-dimensional surfaces in which the levels of green and red fluorescence per cell define the location of cells on a (64 × 64) grid and the frequency

[2] Parks, D. R., R. R. Hardy, and L. A. Herzenberg. Four-color immunofluorescence using phycobiliprotein conjugates on a dual-laser FACS. Manuscript in preparation.

of cells at each location defines the elevation at that location. After interpolating and smoothing this surface, contour lines were drawn to represent equal step changes in the elevation (frequency). Three-color staining data was analyzed by taking "slices" of the histogram generated from one of the stains (for example BLA-1$^+$ cells) and examining the correlated expression of the two other markers (for example IgM and IgD) within this gated population. Such slices are displayed as two-color contour plots. Similarly, four-color staining data was analyzed by gating on a rectangular region of a two-color contour plot for any two immunofluorescences (for example BLA-1$^+$, 2$^+$) and then displaying the two-color contour plot of the other two immunofluorescences (for example IgM and IgD) within this region.

Immunizations and Antibody Responses. Donors were primed with 2×10^7 SE 4 d before sorting and assay. Plaque-forming cell (PFC) estimations were performed by the Cunningham method (34).

In Vitro Cultures. Cells were cultured in RPMI 1640 (Irvine Scientific, Santa Ana, CA) supplemented with 10% fetal calf serum and 5×10^{-5} M 2-mercaptoethanol (2-ME) at 10^6 cells/ml in 1-ml multiwell plates. Lipopolysaccharide (LPS) was added to such cultures at 25 μg/ml and concanavalin A (Con A) was used at 2 μg/ml. Large numbers of blast cells were found in mitogen-treated cultures 2–4 d after stimulation.

Tumor Lines. B cell tumor lines previously characterized for surface marker expression (35) were generously provided by Dr. L. Lanier, Becton-Dickinson Monoclonal Center.

Trypsinization. Spleen cell suspensions prepared as described above were first incubated at 37°C for 10 min in various concentrations of trypsin. The cells were then washed free of enzyme and stained as usual for two-color analysis.

Immunoprecipitation and Analysis. 30-E2 is a rat IgG_{2b} antibody and 53-10.1 is a rat IgG_{2c} antibody. Immunoprecipitations were carried out with staph A after a standard protocol from biosynthetically labeled tumor cell cultures (36). Cells (5×10^6) were labeled for 16 h with 1 mCi of [^{35}S]methionine and then cell extracts were prepared, precleared, and precipitated with antibody-coated staph A. Immunoprecipitated material was then analyzed by two-dimensional polyacrylamide gel electrophoresis, the first dimension to separate according to charge and the second according to molecular weight (36).

Results

Monoclonal Antibodies 30-E2 and 53-10.1 Identify B Cell Subsets. Among a panel of rat anti-mouse spleen cell monoclonal antibodies (21), only two were found to react with a subpopulation of the B cells in spleen. One of these, 30-E2, dully stained ~10–15% of adult BALB/c splenic lymphocytes and was essentially negative on lymph node lymphocytes from the same animal. This pattern held for all strains tested except CBA/N (xid), where ~25–30% of the splenic lymphocytes stained and the mean staining intensity was two- to fivefold higher than that of the positively stained BALB/c cells (Table I). Blocking studies with a monoclonal antibody (14G8) described by Kung et al. (37) that shows similar reactivity patterns suggest that these antibodies recognize identical or closely related determinants. The second monoclonal antibody, 53-10.1, stained ~15% of splenic lymphocytes but only ~1% of lymph node lymphocytes from unimmunized BALB/c mice. This pattern holds for all strains tested, including CBA/N (Table I). We refer to the antigen recognized by 53-10.1 as BLA-1 (B cell lineage antigen) and the antigen recognized by 30-E2 as BLA-2.

We have used two-color immunofluorescence to study the relationships of BLA-1 and BLA-2 with each other and with IgM and IgD. As Fig. 1 shows, BLA-2 is principally on high-IgM cells that are heterogeneous with respect to their IgD expression. Thus, BLA-2 is on the majority of cells that fall within our previously defined B cell populations II and III. BLA-1-bearing cells, in contrast,

TABLE I

BLA-1 and BLA-2 Antigens Are Expressed on Subsets of B Cells

	Percentage of spleen cells stained		
	BALB/c	CBA	CBA/N
IgM^+	50	40	28
$BLA\text{-}1^+/IgM^+$	17	15	16
$BLA\text{-}2^+/IgM^+$	10	15	25*

Spleen cells from 3-mo-old mice were stained with Fl-anti-IgM together with either Bi-anti-BLA-1 or Bi-anti-BLA-2. TR-avidin revealed the biotin reagent staining. The BLA-1 antigen is defined by the 53-10.1 monoclonal antibody. BLA-2 antigen is defined by the 30-E2 antibody. In adult mice, typically 90–95% of the $BLA\text{-}1^+$ or $BLA\text{-}2^+$ cells in spleen are IgM^+ (B cells).

* Level of staining two- to fivefold higher than for normals.

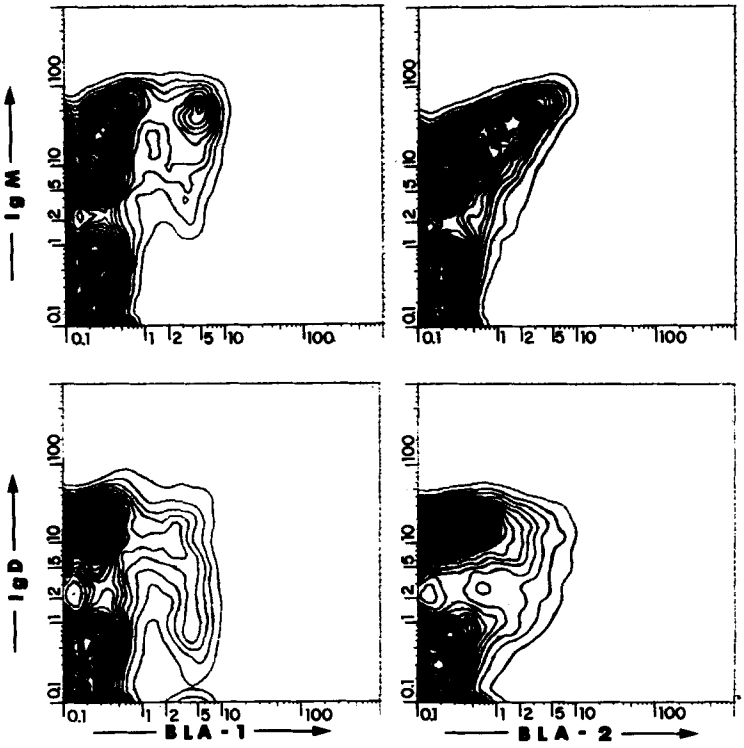

FIGURE 1. 30-E2 and 53-10.1 stain subsets of B cells. Cells were costained with Fl-labeled 30-E2 or 53-10.1 (21) and biotin-coupled anti-IgM (20) or anti-IgD (22); TR-labeled avidin was used as the second step to reveal the biotinated antibody. Our threshold for positive staining was ~1 U for both axes.

are heterogenous for both IgM and IgD expression and therefore cannot be delineated in terms of the previously defined subpopulations. This complex staining pattern led us to apply recently developed three- and four-color immunofluorescence techniques to resolve these populations.

Further Characterization of BLA-1 and BLA-2: Similarities and Differences. Early

on we discovered a number of similarities between the BLA-1 and BLA-2 antigens. Besides being on subpopulations of B cells, both determinants are present on macrophages and erythrocytes, but are never found on T cells in thymus, spleen, lymph node, and peripheral blood (data not shown). BLA-1, however, is on most B cell tumor lines, such as CH1, WEHI-5, and WEHI-259, while BLA-2 is on only a few lines, such as CH1 and WEHI-259 (L. Lanier, unpublished observations and 35). Furthermore, the BLA-1 expressed on spleen cells is very sensitive to trypsinization (as is IgD), while BLA-2 is completely unaffected by this enzyme (Fig. 2). Our analyses indicate expression on lymphocytes, since erythrocytes were eliminated from samples before staining and granular cells were gated out by scatter at 120° (at large angles granular cells scatter considerably more light than lymphocytes).

Immunoprecipitation and two-dimensional gel electrophoresis studies chemically distinguish these antigens (manuscript in preparation). BLA-1 is a single 53 kD acidic polypeptide while BLA-2 is not precipitated from any biosynthetically labeled cell extract. This inability to precipitate the molecule bound by 30-E2 may mean that 30-E2 recognizes a non-protein determinant (for example a glycolipid) or, alternately, that the protein is not well labeled during our incu-

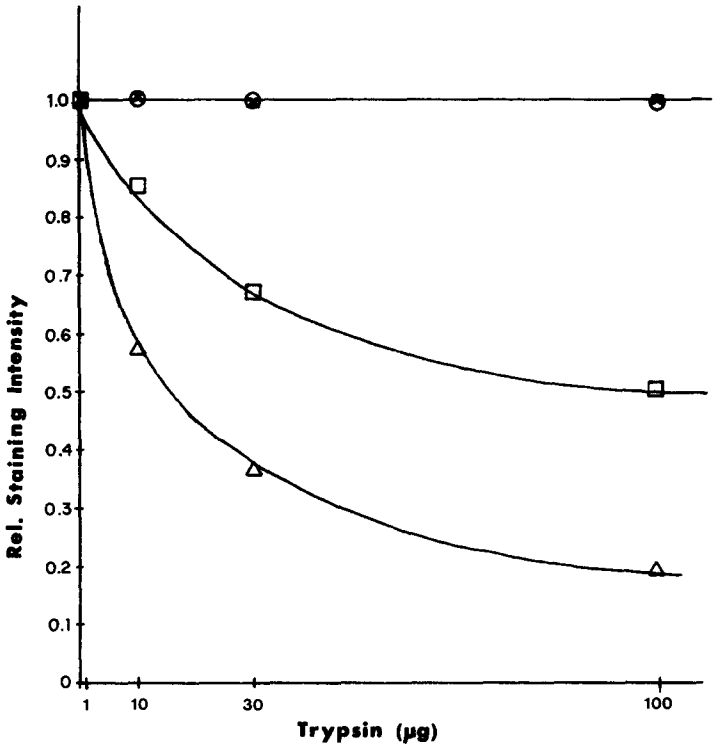

FIGURE 2. BLA-1 (△) and IgD (□) were both trypsin-sensitive while BLA-2 (○) was as trypsin-resistant as IgM (×). 2-mo-old BALB/c spleen cells were treated with indicated amounts of trypsin for 10 min at 37°C and then stained with Fl-anti-IgM and either Bi-anti-BLA-1, Bi-anti-BLA-2, or Bi-anti-IgD. TR-avidin revealed staining with the biotin reagent. The mean staining intensity of the positive cells was normalized (for each stain) to the level found on untreated cells.

TABLE II

Tissue Distribution of BLA-1 and BLA-2 on B Cells: Bone Marrow B Cells Express Both Antigens While Most Lymph Node B Cells Express Neither

	Percent positive cells			
	Bone marrow	Spleen	Peripheral blood lymphocyte	Lymph node
IgM^+	5	50	20	20
$BLA\text{-}1^+/IgM^+$	5	17	3	1
$BLA\text{-}2^+/IgM^+$	5	11	1	0.5

Cells were analyzed as described in Fig. 1. All cell suspensions are from 3-mo-old BALB/c mice. BLA-1 was expressed on many IgM^- cells in bone marrow (typically 70–80%) while BLA-2 was found on fewer IgM^- cells in bone marrow (typically 10–20%).

bation period. We are pursuing the biochemical characterization of the 30-E2 determinant. Recently, we have obtained L cell transfectants that stably express either BLA-1 or BLA-2 which may aid in the biochemical characterization of these molecules.[3] Thus, although there are some similarities in the expression of these two antigens, they are clearly different molecular species.

Tissue Distribution and Developmental Expression. The changes in expression of the BLA-1 and BLA-2 antigens during B cell development suggest that their levels of expression on B cells are inversely related to the degree of B cell maturity (Table II). Analyses of B cells taken from spleen, lymph node, and bone marrow of adult mice show that BLA-1 and BLA-2 are both present on all IgM^+ cells in the bone marrow. In contrast, many IgM^+ cells in spleen lack these antigens and most IgM^+ cells in lymph node lack them. In addition, BLA-1 is present on a majority of bone marrow cells that lack IgM, and, since these cannot all be pre-B cells (38), it appears likely that this antigen is also expressed on cells of non-B lineages. BLA-2, although on fewer IgM^- bone marrow cells, is still on a greater number of cells than can be accounted for as pre-B. Thus, although neither antigens is B lineage specific, their variation in expression on B cells appears related to B cell maturity. We have investigated this relationship here by eliminating IgM^- cells from the analyses and also by eliminating granular cells (defined by scatter at large angles).

Similar studies with spleen cells from young mice (Table III) support the idea that BLA-1 and BLA-2 are primarily expressed on immature or precursor B cell populations. Most of the IgM^+,IgD^+ and IgM^+,IgD^- B cells in spleen expressed both BLA-1 and BLA-2 at 2 wk of age. As the mouse matured, the frequency of BLA-1- and BLA-2-bearing cells among total splenic B cells decreased. This decrease appears to be due to the emergence of B cell population I in the weanling animal, since the frequency of population I cells reached stable adult levels by 2–3 mo of age and the frequency of $BLA\text{-}1^+$ and $BLA\text{-}2^+$ cells reached a stable minimum at about the same time.

Three-Color Immunofluorescence Data Demonstrate that Different Subpopulations of

[3] Hsu, C., P. Kavathas, and L. A. Herzenberg. Expression of cell surface antigens on mouse L cells transfected with DNA from nonexpressing cells. Manuscript submitted for publication.

TABLE III

All Splenic B Cells from Immature Animals Express BLA-1 and BLA-2

	Percent positive cells in BALB/c mice aged:			
	2–5 d	14 d	28 d	56 d
IgM^+	5	15	30	50
$BLA\text{-}1^+/IgM^+$	5	15	30	15
$BLA\text{-}2^+/IgM^+$	5	10	10	10

BLA-1 is also expressed on many IgM^- spleen cells in young animals. For example, 90% of spleen cells from 2–5-d-old animals are $BLA\text{-}1^+$.

B Cells Are Missing from xid and Nude Mice. Since these antigens are present on non-B cells, two-color analysis of the correlated expression of BLA-1 and BLA-2 on B cells was always obscured by the variable numbers of non-B cells expressing one or both antigens. Accordingly, we used three-color immunofluorescence to study the relationship of BLA-1 and BLA-2 on IgM^+ cells. Direct comparison of the correlated expression of BLA-1 and BLA-2 for CBA, CBA nu/nu, and CBA/N on B cells (IgM^+) clarifies the changes in B cells resulting from the xid and nude defects. As is shown in Fig. 3, all four possible types of splenic B cells were found in CBA by three-color analysis: $BLA\text{-}1^+,2^+$ cells, $BLA\text{-}1^-,2^-$ cells, $BLA\text{-}1^+,2^-$ cells, and $BLA\text{-}1^-,2^+$ cells.

The frequencies of the four BLA B cell types in normal, xid, and nude mice are summarized in Table IV. The major difference found is between CBA and CBA/N: the latter lacked $BLA\text{-}1^+,2^-$ cells. A second distinction is that CBA/N mice had fewer of the $BLA\text{-}1^-,2^-$ (double-negative) B cells that constitute the predominant mature population in normal mice. In contrast, nude mice had fewer $BLA\text{-}1^-,2^+$ cells and had more of the double negative cells (than normals). A striking finding is the similarity in the three strains of mice in the IgM-staining histogram for each BLA-1,BLA-2 type. That is, the $BLA\text{-}1^-,2^+$ B cells had high levels of surface IgM whereas the $BLA\text{-}1^-,2^-$ B cells had lower levels.

Four-Color Immunofluorescence Data Provide Evidence that the Correlated Expression of BLA-1 and BLA-2 Delineates Two Distinct Lineages of B Cells. The similarity of IgM expression on particular BLA-1,BLA-2 cell types led us to investigate the correlated expression of IgM and IgD within these four kinds of B cells using four-color immunofluorescence measurements. Such analyses comparing normal, xid, and nude splenic B cells demonstrated striking similarities in the correlated expression of IgM and IgD among the B cells that are $BLA\text{-}1^-,2^+$ in all three animals (Fig. 4). In addition, the similarities and differences in the IgM,IgD plots for the four BLA-1,BLA-2 types suggest relationships among these four kinds of B cells. Specifically, the $BLA\text{-}1^-,2^+$ cells had a correlated expression of IgM and IgD similar to that of a portion of the $BLA\text{-}1^+,2^+$ cells, and the $BLA\text{-}1^-,2^-$ cells had a correlated expression of IgM and IgD similar to the $BLA\text{-}1^+,2^-$ cells. These cells ($BLA\text{-}1^+,2^-$) in turn had a correlated expression of IgM and IgD similar to a portion of the $BLA\text{-}1^+,2^+$ cells. These similarities in IgM,IgD expression together with the differences observed in mice with genetic defects (xid and athymic) are consistent with there being two B cell developmental pathways (lineages).

BLA-1 and BLA-2 Are Expressed on Antigen-stimulated B Cells. Thus far we have

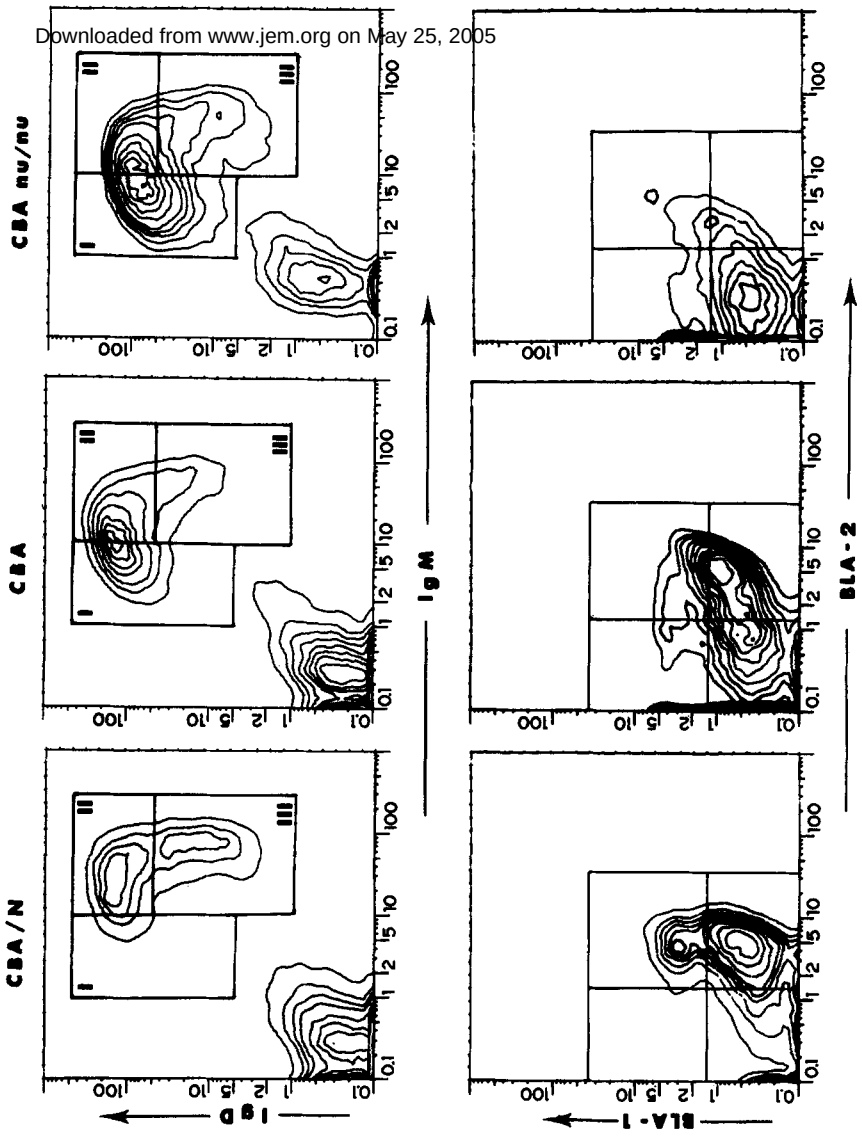

FIGURE 3. Three-color immunofluorescence demonstrates that BLA-1 and BLA-2 together divide splenic B cells into four populations of cells, two of which are altered in xid mice. (*Bottom*) Cells were stained with AP-anti-BLA-1, Bi-anti-BLA-2, and F1-anti-IgM. PE-avidin revealed the BLA-2$^+$ cells. IgM$^+$ CBA splenic B cells fell in every quadrant of the BLA-1/BLA-2 two-color contour plot. CBA/N B cells, on the other hand, lacked BLA-1$^+$,2$^-$ B cells and had greatly diminished numbers of double-negative B cells. In contrast, CBA nude mice had diminished numbers of BLA-1$^-$,2$^+$ B cells and increased numbers of the two populations affected in xid mice. (*Top*) Comparison two-color IgM/IgD staining for the same cell suspensions (with Fl-anti-IgM, Bi-anti-IgD; TR-avidin as second step). The regions previously defined as populations I, II, and III are marked on the plots.

TABLE IV

BLA-1 and BLA-2 Together Define Four Types of B Cells, One of Which Is Missing from CBA/N and Another That Is Found at Lower Frequency in Nude Mice

	Percentage of splenic B (IgM^+) cells		
	CBA/N	CBA	CBA nu/nu
BLA-1^-,2^-	30	45	66
BLA-1^-,2^+	51	25	9
BLA-1^+,2^-	1	14	13
BLA-1^+,2^+	17	16	12

All animals were between 3 and 6 mo of age. All cells were gated on IgM^+ cells since, for example, BLA-1^-,2^- cells in spleen included all the T cells if not so gated. The boundaries used for determining percentages of cells in the four populations are drawn on the BLA-1/BLA-2 plots in Fig. 2. Percentages are expressed out of total IgM-bearing cells, which are as follows: CBA/N, 30% of total spleen cells; CBA, 45%; CBA nu/nu, 80%. All values are averages of at least five determinations of different animals, with deviations typically 5–10%.

described results obtained with animals that were not specifically immunized and that appeared generally healthy. We specifically immunized animals to investigate the changes in BLA-1 and BLA-2 expression on B cells that occur upon immunization. As we showed earlier, lymph node B cells were essentially negative for both markers. After immunization, ~5–15% of lymph node cells expressed both markers, and (as is shown in Fig. 5) we found that such cells also stained brightly for the germinal center B cell marker, PNA (28, 39). Serial sections of immunized lymph node tissue (Fig. 5) confirmed that BLA-1 staining delineates germinal centers (defined by PNA) in immunized lymph node.

Since germinal center cells expressed both BLA-1 and BLA-2, we next investigated the antibody-forming cells (derived from germinal center cells) for BLA-1,2 expression. To do this, we sorted spleen cell suspensions obtained from immunized animals into BLA-negative and -positive fractions and carried out plaque assays on the sorted cells. As shown in Table V, BLA-1^+ cells included both IgM and IgG PFC whereas BLA-2^+ cells included only the IgM PFC. Furthermore, adoptive transfer memory assays showed that BLA-1^+ cells included memory cells 6 d after immunization, but did not include memory cells 6 wk after immunization (data not shown). Thus, memory cells initially expressed BLA-1, but lost this marker soon after generation.

Reexpression of BLA-1 and BLA-2 on LPS Blast Cells. To more carefully study the appearance of these antigens on activated cells, we analyzed spleen cell cultures treated either with a B cell mitogen (LPS) or a T cell mitogen (Con A). The data in Fig. 6 show that BLA-1 and BLA-2 expression was induced on LPS blasts (defined by forward scatter). In addition, as is summarized in Table VI, Con A blasts expressed neither of these antigens. Thus, BLA-1 and BLA-2 are not simply "blast" antigens that appear on any dividing cell. The kinetics of appearance and disappearance of BLA-1 and BLA-2 are currently being investigated more thoroughly both in vitro and in vivo. FACS experiments clearly demonstrate that sorted BLA-1^- cells cultured in the presence of LPS express

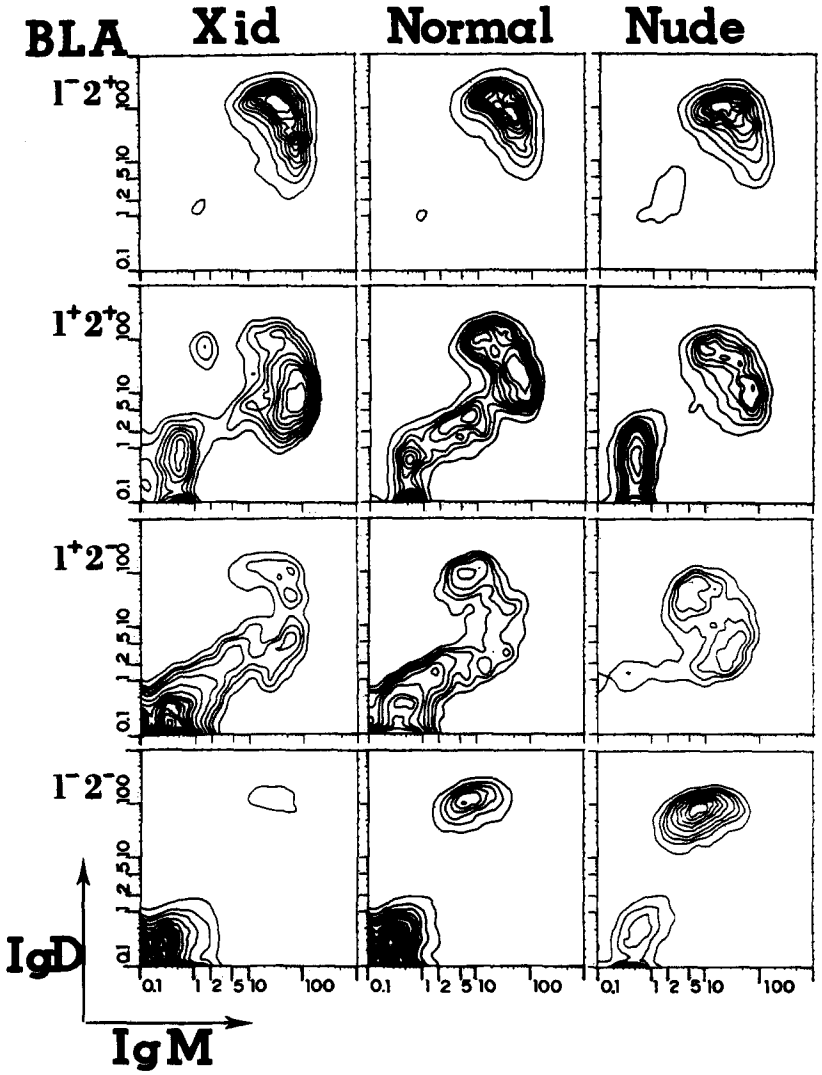

FIGURE 4. Four-color immunofluorescence revealed distinct IgM/IgD staining patterns for the four populations defined by the expression of BLA-1 and BLA-2. Cells were stained with F1-anti-IgM, Bi-anti-IgD, PE-anti-BLA-2, and AP-anti-BLA-1. TR-avidin revealed the IgD^+ cells. The plots are arranged to correspond to the BLA-1/BLA-2 differentiation model proposed in the Discussion and are diagrammed in Fig. 6: the "terminal" B cell populations for the two B cell lineages are at top (BLA-2 only) and bottom ($BLA\text{-}1^-,2^-$).

both antigens (data not shown). In addition, lymph node B cells (few of which expressed either BLA-1 or BLA-2) gave rise to large numbers of $BLA\text{-}1^+,2^+$ blasts when cultured for 4 d with LPS. Thus, expression of BLA-1 and BLA-2 can be induced on mature B cells that initially bear neither markers, by activating these cells either specifically with antigen or nonspecifically with the polyclonal B cell mitogen, LPS.

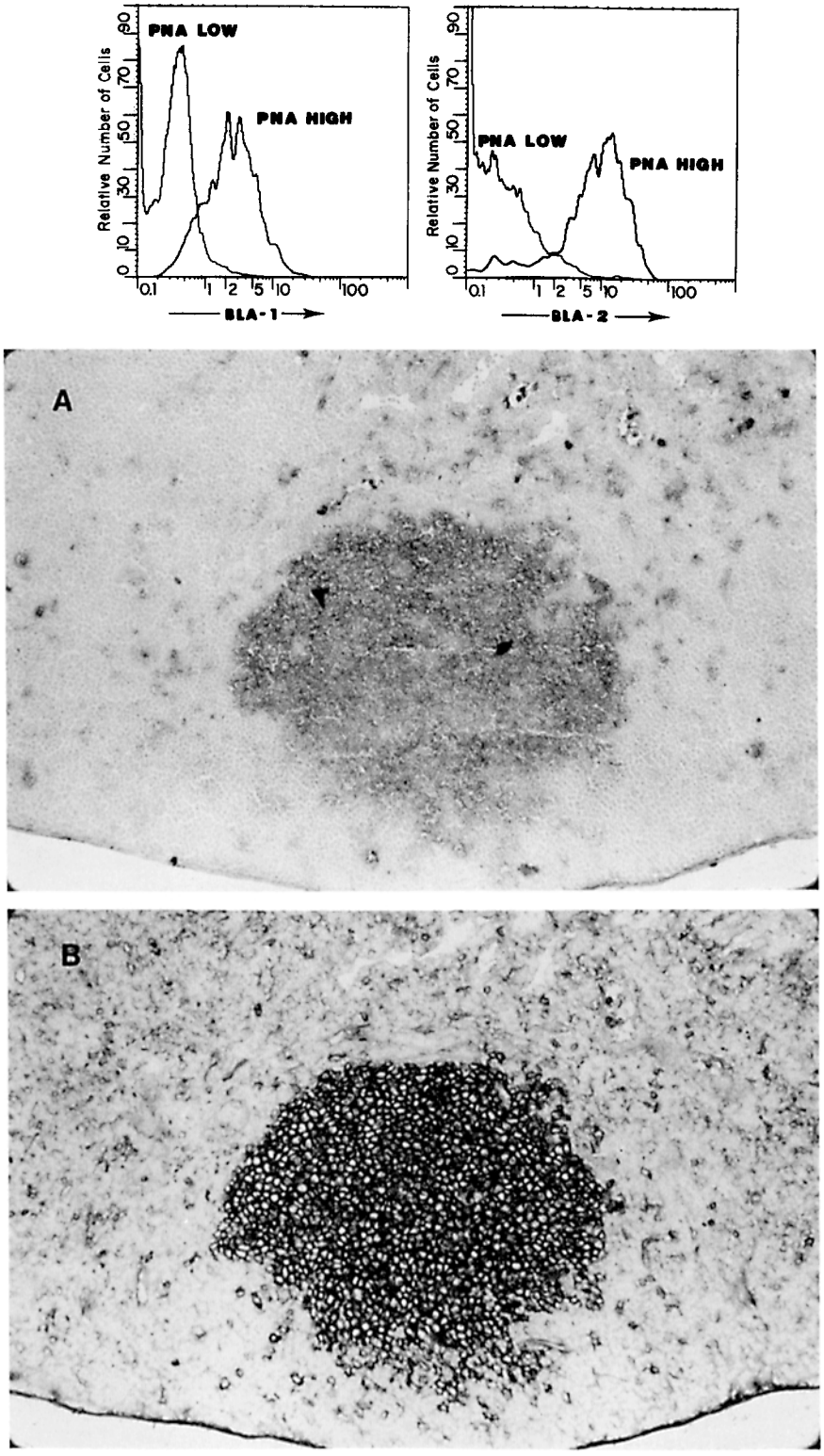

FIGURE 5. BLA-1 and BLA-2 were both expressed on germinal center cells. A cell suspension of immunized lymph node cells was incubated with TR-PNA together with either Fl-anti-BLA-1 or F1-anti-BLA-2. Essentially all PNA-high cells were germinal center cells; such cells also stained for BLA-1 and BLA-2. IgM PFC (which are predominantly PNA^-) also stained for BLA-1 and BLA-2. Tissue section staining with BLA-1 clearly defined germinal centers in an immunized lymph node. Frozen serial sections of a lymph node taken from an SE-immunized BALB/c mouse were stained either with Bi-anti-BLA-1 (*A*) or Bi-PNA (*B*) followed by horseradish peroxidase-conjugated avidin. Incubation with 0.1% diaminobenzidine in 0.3% hydrogen peroxide in phosphate-buffered saline visualized the staining.

TABLE V

IgM PFC Carry Both BLA-1 and BLA-2 While IgG PFC Carry only BLA-1

	IgM PFC (1°)		IgG PFC (2°)	
	Exp. 1	Exp. 2	Exp. 3	Exp. 4
	%			
BLA-1$^+$	98	97	98	100
BLA-1$^-$	2	3	2	0
BLA-2$^+$	100	100	3	8
BLA-2$^-$	0	0	97	92

The values reported are the percentages of antigen-specific PFC that fall within a particular range of BLA-1 or BLA-2 expression. Experiment 1 (dinitrophenyl-keyhole limpet hemocyanin [D-K] immunization) gave ~1,000 IgM PFC/spleen; experiment 2 (trinitrophenyl-Ficoll) gave ~100,000 IgM PFC/spleen; experiment 3 (D-K, D-K) gave 70,000 IgG PFC/spleen; and experiment 4 (D-K, D-K) gave ~30,000 IgG PFC/spleen. IgM PFC in experiments 3 and 4 were both ~500 PFC/spleen. Staining did not significantly alter the percentage of PFC per spleen.

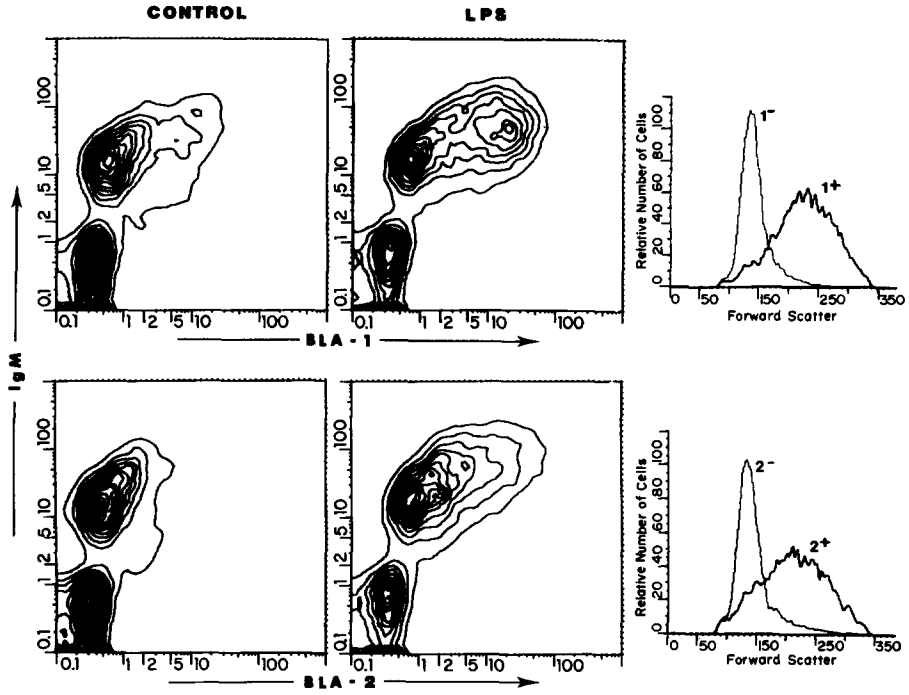

FIGURE 6. Expression of BLA-1 and BLA-2 was induced on LPS blasts. Spleen cells were ltured with 25 μg/ml LPS for 4 d and then stained with Bi-anti-IgM and Fl-anti-BLA-1 or Fl-anti-BLA-2. TR-avidin was used to reveal the IgM staining. LPS blasts were defined by scatter. A small number of blasts were BLA-2$^-$. The scatter profiles of BLA-1 and BLA-2 positive and negative cells in the LPS culture clearly demonstrate that the blast cells express both antigens.

TABLE VI

BLA-1 and BLA-2 Are Expressed on B Blasts But Not on T Blasts

	Percent positive cells after:	
	LPS culture	Con A culture
BLA-1$^+$	40*	1
BLA-2$^+$	40*	<1
IgM$^+$	70	15
Thy-1$^+$	27	85*

Spleen cells from 3-mo-old BALB/c mice were cultured for 2 d either in 25 μg/ml LPS or 2 μg/ml Con A.

* These fractions consist predominantly (>95%) of large (by scatter) cells.

Discussion

Data presented here comparing the correlated expression of BLA-1 and BLA-2 on CBA, CBA nu/nu, and CBA/N spleen cells demonstrate the existence of four types of B (Ig$^+$) cells. One of these lacked both BLA-1 and BLA-2 and included the predominant recirculating resting B cell population in normal animals; the second expressed both antigens and appears to include B cells that migrated most recently from bone marrow to spleen (and have not as yet lost BLA-1 or BLA-2). The latter cell type (BLA-1$^+$,2$^+$) was present in the spleens of all three strains of mice at similar levels, whereas the former (BLA-1$^-$,2$^-$) was found at considerably reduced frequency in CBA/N mice.

Developmental expression and tissue distribution support the idea that BLA-1$^+$,2$^+$ cells in the spleen include the most immature B cells (constituting a "precursor" population) and that the BLA-1$^-$,2$^-$ cells include the most mature B cells (constituting a "terminal" population) that will rest as such unless they are stimulated with antigen. That is, bone marrow B cells express both antigens, whereas few lymph node B cells (considered to be most "mature") express either antigen. Furthermore, all splenic B cells in the neonate express both BLA-1 and BLA-2, and the decrease in the percentage of B cells expressing these antigens coincides with the increase of population I (the mature resting B cells) that occurs between 1 and 2 mo of age.

Two other BLA-1, BLA-2 B cell types were present in normal mouse spleen: one expressed BLA-1 but lacked BLA-2 and the other expressed BLA-2 but lacked BLA-1. The former population (BLA-1$^+$,2$^-$) was greatly diminished (tenfold or more) in xid mice while the latter (BLA-1$^-$,2$^+$) was reduced (by two- to threefold) in athymic (nude) animals. The decrease in both BLA-1$^+$,2$^-$ and BLA-1$^-$,2$^-$ cell types in xid mice and the similarity in the expression of IgM on both prompts us to suggest that the BLA-1$^+$,2$^-$ cells represent cells that are in transition from the immature (precursor) BLA-1$^+$,2$^+$ stage to the mature (terminal) BLA-1$^-$,2$^-$ resting B cell stage. The changes in frequency that occur in the BLA-1$^+$,2$^-$/BLA-1$^-$,2$^-$ types contrast with the changes that occur in the BLA-1$^-$,2$^+$ cells. This is consistent with the concept of two distinct B cell lineages: xid mice possess the lineage that expresses BLA-2 alone, but lack the lineage that expresses BLA-1 alone (and derivative double negative cells), whereas nude mice have increased numbers of the lineage that expresses BLA-1 alone (and double negatives) and fewer cells of the lineage that express BLA-2 alone.

Four-color analysis permitted us to examine the correlated expression of IgM and IgD for these four BLA-1,BLA-2 B cell populations, and such analysis supports the hypothesis that these antigens distinguish separate lineages of B cells. The B cells that bear BLA-2 alone had a very different correlated expression of IgM and IgD compared with the B cells that bear neither BLA-1 nor BLA-2. Similarities and differences in the IgM,IgD expression on these four populations lead us to propose a model for B cell differentiation in the spleen, shown schematically in Fig. 7. Thus, we suggest that there are two "mature" populations of B cells in the spleen of normal mice, one that has neither BLA-1 nor BLA-2 (and constitutes the majority of the recirculating B cell pool) and the other that has BLA-2 alone (residing mainly in the spleen). These correspond roughly to B cell populations I and II, which we defined previously on the basis of the correlated levels of IgM and IgD. The lack of the BLA-1^+,2^- lineage (and its resulting BLA-1^-,2^- population) results in the aberrant IgM,IgD profile in xid mice that led us to initiate these studies.

In this model of B cell differentiation pathways, we show the BLA-1^+,2^+ population as a single cell that branches to give two pathways. With four-color analysis, there is a suggestion of heterogeneity within this population: such cells from normal and nude mice have one population that expressed low IgM, high IgD levels and a second that expressed high IgM, low IgD levels; such cells from CBA/N mice have many fewer cells of the low IgM, high IgD phenotype (Fig. 4). This suggests that the two lineages of B cells are distinct as early as the BLA-1^+,2^+ stage, and that xid mice lack one lineage even at this stage of differentiation.

Previous studies (16, 17) have suggested that xid and nude animals lack different lineages of B cells. CBA/N mice clearly have a deficit of the mature B cells found in normal animals and have a constellation of functional impairments associated with this defect, including a failure to respond to certain T-independent (TI-2) antigens (10–12). Nude mice fail to respond to T-dependent antigens; however, it is difficult to ascertain whether this failure is due entirely to the absence of T cells that control the further (antigen-driven) differentiation of

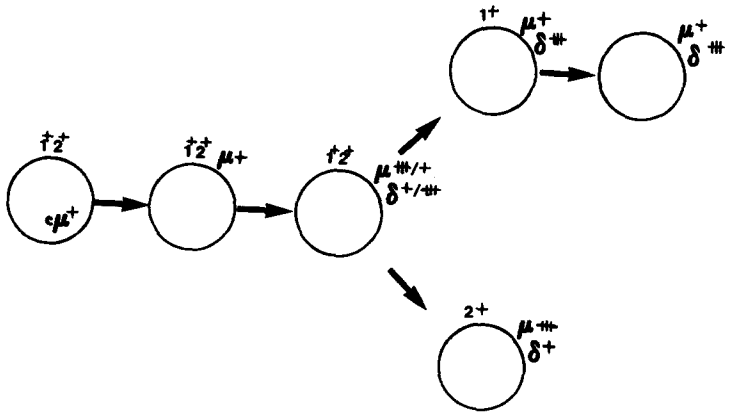

FIGURE 7. Model of B cell differentiation pathways showing expression of IgM, IgD, BLA-1 (labeled 1) and BLA-2 (labeled 2). (+++) A high surface level of the particular antigen; (+ and +++) very heterogenous level of the particular antigen.

mature resting B cells or whether the immune defect in these mice also includes an impairment of B cell differentiation that results in an altered population of virgin B cells.

A two-lineage model of B cell development has interesting implications, considering the antigen response defects in nude and xid animals. Since xid mice do not respond to TI-2 type antigens, the missing lineage in these mice would appear to be required for response to such antigens. Previous studies (13–15) demonstrating that CBA/N mice lack Lyb-3^+,5^+ B cells suggest that the BLA-1^+,2^- B cells (and the mature BLA-1^-,2^- cells that derive from them) constitute this population. Nude mice, in contrast, have fewer cells of a different putative lineage (BLA-1^-,2^+) that may require either a specific differentiating environment (absent from nude mice) or direct stimulation from T cells in order to appear.

If we accept that the majority of the B cells in spleen and lymph nodes are derived from BLA-1^+,2^+ cells (recently emigrated from bone marrow), then LPS clearly triggers BLA-1 and BLA-2 reexpression since (as we have shown) sorted BLA-1^- splenic B cells cultured with LPS give rise to BLA-1^+ blast cells. Furthermore, specific antigen stimulation apparently triggers a similar reexpression (of BLA-1 and BLA-2) since germinal center cells are BLA-1^+,2^+ and most likely are derived from the predominant B cell population (BLA-1^-,2^-) after immunization with T-dependent antigens. The presence of both BLA-1 and BLA-2 on IgM-secreting PFC also supports this hypothesis.

The distinction that BLA-2 makes between IgM- and IgG-secreting cells is striking and suggests that BLA-2 is lost as B cells differentiate further toward IgG secretion. Similarly, evidence that long-term resting memory B cells (6 wk after priming) do not express BLA-1 whereas the majority of short-term memory cells (5–7 d after priming) do express BLA-1 indicates that antigen-stimulated BLA-1 expression can also be transient. Thus, these antigens may be gained and lost several times during B cell differentiation.

These findings raise the question of whether BLA-1 and BLA-2 are B cell blast antigens that appear on the cell whenever it leaves the resting stage (G_0) of the mitotic cycle and are lost whenever it returns to the resting state. Our findings rule out this explanation in its simplest form since BLA-1 and BLA-2 are expressed independently on individual cells and thus must be regulated by independently operating mechanisms. In addition, the reexpression of these antigens does not simply represent the entry of resting (G_0) cells into mitosis since end-stage IgM-secreting cells (PFC) continue to express both antigens.

Kung et al. (37) showed that a monoclonal antibody called 14G8 (which apparently recognizes BLA-2) stains the majority of CBA/N B cells, but they concluded (rightly) that this antibody alone is insufficient to clearly resolve a lineage of B cells present in normal mice but missing in CBA/N. Similarly, although the correlated expression of IgM and IgD defines a population of B cells that is greatly diminished in CBA/N mice (1–3), any attempt to precisely distinguish this population on the basis of IgM and IgD expression fails because the amounts of surface Ig on the BLA-1^-,2^+ population (present in CBA/N) overlaps the amounts on the BLA-1^+,2^- population (missing from CBA/N).

In conclusion, BLA-1 and BLA-2 together divide B cells into several distinct

functional subpopulations and probably define two distinct lineages of B cells (one missing in CBA/N, the other depleted in nude mice). These populations/lineages, which are both present in normal mice, roughly correspond to the so-called Lyb-3,5$^+$ and Lyb-3,5$^-$ B cell populations; however, we are wary of this notation since Lyb-3,5$^-$ cells are currently defined as the B cells present in CBA/N mice and are not necessarily equivalent to any population of B cells in normal mice (40).

Much work remains to correlate the expression of these B cell surface antigens with functionally defined B cell subpopulation properties (41), to demonstrate the lineage relationships among these populations, and to explain the genetic defects known for B cells. The findings to date, however, clearly demonstrate that caution is required in interpreting the results of functional studies conducted with unfractionated splenic B cells, even if such studies compare normal with xid B cells (42). In a sense, such studies are inadvisable since they could well be akin to studying T cell function without recognizing the distinction between helper and suppressor subsets of T cells.

Summary

Subpopulations of mouse B cells express different amounts of two antigens (BLA-1 and BLA-2) recognized by rat monoclonal antibodies (53-10.1 and 30-E2). Two-color immunofluorescence analysis on the fluorescence-activated cell sorter (FACS) shows that the 53-10.1 monoclonal antibody reacts with a similar proportion of splenic B cells from normal and CBA/N (xid) mice, whereas 30-E2 reacts with most CBA/N B cells but with only a fraction of normal B cells. Data from three- and four-color immunofluorescence analyses with xid, athymic (nude), and normal mice suggest that the order in which these antigens are lost during B cell differentiation distinguishes two B cell lineages: immature B cells express both antigens, intermediate-stage B cells of one or the other lineage express only BLA-1 or only BLA-2, respectively, and mature resting B cells express neither. CBA/N mice lack one of the putative intermediate populations (BLA-1$^+$,2$^-$); thus, this population apparently gives rise to the predominant mature B cell population, which is present in normal adult spleen and lymph node but is missing in CBA/N. The other putative intermediate population (BLA-1$^-$,2$^+$) is decreased by two- to threefold in spleens from nude mice compared with strain-matched controls.

Both BLA-1 and BLA-2 antigens rapidly reappear after specific (antigen) or nonspecific (lipopolysaccharide) B cell activation. IgM plaque-forming cells (PFC) derived from such activated cells continue to express both antigens while IgG PFC express only BLA-1.

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